Fast multiplex analysis of antibodies in complex sample matrix using the microfluidic Evalution™ platform

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Abstract

Biosensor development requires comprehensive research for establishing the optimal bioassay conditions that determine the final kinetics, sensitivity and specificity. Different systems have been developed to study bioreceptor-target interactions but they often have drawbacks, such as long hands-on time, low throughput, high sample consumption and high cost. In this work, the potential of the novel microfluidic Evalution™ platform has been evaluated for developing sandwich-based assays in a fast and high-throughput fashion. An immunoassay for the detection of influenza A nucleoprotein was used as a model system. Exploiting the platform's unique features, various typical bioassay parameters (e.g. aspecific binding between assay components, different reagent concentrations and incubation times) were tested for three capture antibodies in a simple and fast manner (2 runs of 80 min). The selected conditions, giving the highest signal-to-noise ratio, were directly employed on the same platform to detect nucleoprotein in buffer and nasopharyngeal swabs. Two antibodies with a higher dissociation constant (Ab11 and Ab12) required longer incubation times (60 min) for sensitive detection (limit of detection (LOD) of 0.48 and 0.26 ng mL^{-1}, respectively) compared to an antibody with lower dissociation constant (LOD of 0.04 ng mL^{-1} for Ab66 within 30 min). Moreover, one antibody (Ab12) showed limited capacity to capture nucleoprotein directly in sample matrix. The obtained results were in accordance with previous studies performed on an ELISA and SPR platform with the same antibodies. This positions the Evalution™ platform as a reliable platform for fast and multiplex analysis of antibodies' performance both in buffer and complex sample matrices.

Keywords

Bioassay development, Barcoded microparticles, Microfluidic Evalution platform, Multiplex analysis, Influenza nucleoprotein

1. Introduction

Bioassay development is one of the most crucial aspects for obtaining a functional biosensor and requires a methodological approach to select the ultimate assay components and conditions. Specifically, the bioreceptors have a major influence on the final assay performance since they determine not only the kinetics of the binding reaction but also the sensitivity and specificity of the interaction. Moreover, their performance in complex sample matrices defines the applicability of the developed bioassay in real samples. Ideally, receptors should bind to the target with fast kinetics, while discriminating it from other abundantly present sample components. To achieve this, multiple bioreceptors need to be tested for their interaction with the target under different conditions in order to select the most prominent candidate for the assay.

Different techniques are available for the analysis of bioreceptor-target interactions, with enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR)[1,2] being most commonly used. In a typical ELISA, the target is sandwiched between two antibodies and an enzyme is used to generate signal[3]. Although simple in its format while providing high throughput, standard ELISAs require significant handling time, have long time-to-result (because the different incubation times are largely dependent on diffusion), have high sample consumption and provide only endpoint measurements[1]. SPR is an alternative label-free technique used to monitor interactions of molecules (e.g. protein-protein, DNA-protein). Although it allows real time analysis in a short assay time, standard commercial SPR devices (e.g. Biacore) still have a low throughput, due to the limited number of flow channels, and low multiplexing capacity. Moreover, the measurements usually have a large reagent consumption, thereby increasing the assay cost and requiring a larger sample volume[2].

During the last couple of years, several methods have been developed to overcome the above mentioned disadvantages of the standard ELISA and SPR platforms. Thus, the size of bulky prism-based SPR instruments has been reduced, resulting in less sample consumption and decreased equipment and analysis cost. However, this comes at a price since the sensitivity of these systems is generally lower compared to the standard SPR systems[4–7]. Other techniques providing bioreceptor-target interaction analysis, such as dynamic force spectroscopy and microscale thermophoresis, consume limited amount of sample and allow single molecule analysis but have a very low throughput[8,9]. Recently, significant effort has been invested in developing platforms with high multiplexing capacity since it has a major influence on the total assay time and cost of the analysis system[3]. One of the most commonly used is the xMAP technology of Luminex. Here, magnetic beads are color coded with specific fluorophores and coated with antibodies to capture the target, which leads to signal generation that can be detected using flow cytometry or CCD imaging. Multiple sandwich immunoassay kits using the xMAP technology are commercially available[10]. Another possibility is printing multiple antibodies on specific spots on an antibody array and analyzing the interaction of each target with their corresponding spot (e.g. RayBiotech, Quansys Biosciences)[11,12]. Although these techniques allow high multiplex analysis, they still require significant hands-on time. To reduce the handling time and sample consumption, some microfluidic platforms have been successfully integrated with standard ELISA but have only limited multiplexing[13,14].

Recently, a new platform called Evalution™ (MyCartis NV, Belgium) has been launched that uses barcoded microparticles (µPs) in a fully automated microfluidic environment. While this platform combines fast assay time with a high level of multiplexing (up to 150-plex in one channel and the possibility to run up to 16 channels simultaneously), it also reduces sample consumption because the flow in the channels is generated by a pressure difference, thus avoiding the use of tubing. The instrument provides dynamic control over assay conditions by allowing real-time data processing and display. Because of all these features, Evalution™ has the potential to be used not only for bioassay development but also for target detection. Although the standard ELISA and SPR systems can also be

used as diagnostic tools[15–18], the ongoing trends in the diagnostic field towards point-of-care tests and personalized medicine reduce their usability in those fields[19]. The first example showing the capacity of the Evalution™ platform as a diagnostic tool was the sensitive detection of cytokines in a 9-plex format[20].

In this work, the Evalution™ platform has been evaluated for its potential for developing sandwich-based assays in a fast and high-throughput fashion both in buffer as well as in complex sample matrix. A sandwich immunoassay for the detection of influenza A nucleoprotein is used as a model system. Previously, our group has used the same model on ELISA and SPR platforms[21] and hence these results can be used to benchmark the results obtained on Evalution™. Using the possibility to test multiple parameters in a single run, several analyses were performed to: (i) monitor the aspecific interaction between assay components and increase the signal-to-noise ratio, which is crucial in bioassay optimization; (ii) test the effect of varying incubation times of the nucleoprotein and (iii) test the assay performance using a complex sample matrix, namely nasopharyngeal swabs.

2. Material and methods

2.1. Reagents

All reagents were purchased from Sigma-Aldrich (Belgium) unless stated otherwise. Based on previously published work[21], three commercially available monoclonal mouse antibodies (ab110661, ab128193 and ab66191) were selected for analyzing their binding affinity towards recombinant influenza A nucleoprotein, in this manuscript referred to as Ab11, Ab12 and Ab66, respectively. All monoclonal antibodies were raised against nucleoprotein isolated from real virus and purchased from Abcam plc. (United Kingdom). Polyclonal rabbit antibody against nucleoprotein (11675-RP01, referred to as secondary antibody) was purchased from SinoBiological Inc. (China). Biotinylated goat-anti-rabbit antibody (A16114, referred to as detection antibody) was obtained from Thermo Fisher Scientific Inc. (United States). Streptavidin labeled with phycoerythrin (SAPE-001, referred to as SA-PE) and buffer diluent (PECD-100) were purchased from Moss Inc. (United States).

LowCross buffer (100 500) was obtained from Candor Bioscience GmbH (Germany). Custom made recombinant influenza A nucleoprotein (IMR-274) was purchased from Imgenex (United States).

2.2. Evalution™ instrument

The Evalution™ platform relies on three major components, being barcoded µPs, microfluidic assay cartridges and an instrument for the integration of all assay steps[20]. The µPs are silicon discs with a diameter of 40 µm and a thickness of 10 µm. They are barcoded with 10 binary coding bits on their periphery, enabling 1024 (2^{10}) different codes. The central area is functionalized to allow coupling of biomolecules and is dedicated to measure fluorescence intensity. By the immobilization of different capture molecules onto differently encoded µPs, mixed µP populations can be prepared, creating a multiplex environment. These µPs are loaded into a microfluidic cartridge consisting of 16 microfluidic channels. Once the cartridge is inserted into the instrument, liquid transport is achieved by applying a pressure difference between the inlet and outlet of the microchannels (a more detailed description of this technology can be found in Falconnet et al., 2015[20]). Prior to each run, all channels are scanned to determine the background fluorescence signal. After capturing the analyte on the µP surface, a detection partner (either antibody or aptamer) labeled with a fluorophore is bound. Finally, the channels are scanned optically to identify the barcode of each µP and measure its corresponding fluorescence. All assay steps (incubation, washing, detection) are performed within the instrument. Moreover, it exhibits a temperature control between 25 °C and 95 °C, allowing also DNA hybridization analysis.

2.3. Functionalization of microparticles

The lyophilized µPs (MyCartis NV, Belgium) were resuspended in 200 µL molecular biology grade water. The liquid was immediately aspirated and transferred to an Eppendorf Protein LoBind tube containing 100 µL activation solution (100 mM MES, 0.3 % Tween 20, pH 3.5). Any remaining µPs were transferred following the same procedure. Next, the µPs were washed three times with 500 µL activation solution. Next, the carboxylated µPs were activated using EDC/NHS chemistry. Briefly, a volume of 600 µL

activation solution containing 38 mM sulfo-NHS and 43 mM EDC was added. The µPs were homogenized by vortexing shortly and placed on a rotator mixer for 1 h. After incubation, the µPs were washed three times with activation solution and three times with coupling solution (100 mM MES, 0.3 % Tween 20, pH 5.4). Activated µPs were suspended in 520 µL coupling solution and 80 µL of 0.5 mg mL^{-1} protein solution in PBS (either Ab11, Ab12 or Ab66) was added. The µPs were vortexed and incubated for 1 h on the rotator mixer. Finally, the µPs were washed three times in storage buffer (10 mM PBS, 0.3 % Tween 20, pH 7.4) and resuspended in 500 µL of the same buffer. Aliquots, each containing 2500 µPs, were prepared in Protein LoBind tubes by adding 62.5 µl of the functionalized particles to 100 µL storage buffer and stored at -20 °C until further use.

2.4. Loading of the microfluidic cartridge with the functionalized microparticles

Loading was performed in a semi-automated fashion using a cartridge loading station that interfaces with the cartridge (MyCartis NV, Belgium)[20]. Ab11, Ab12 and Ab66 were coupled to different populations of µPs using the protocol described in section 2.3, each representing a distinct population. A µP mix was prepared by mixing together one aliquot (2500 µPs) of each population and non-functionalized control µPs in storage buffer to a final concentration of 5000 µPs mL^{-1}. The microfluidic cartridge was inserted into the loading station. Prior to the loading, the inlet wells were filled with 20 µL ethanol and the channels were primed by flowing for 2 seconds to wet the channels. Next, the ethanol was replaced by 110 µL storage buffer and a second priming step was performed. Following, 100 µL of the µP mix was loaded to each channel until reaching 1 mm of loading length, which corresponds to approximately 39 µPs per population. Finally, the solution in the inlet wells was replaced with 100 µL storage buffer before starting the assay run.

2.5. Signal-to-noise ratio optimization

Two optimization experiments were performed for the different assay steps involved in the detection of nucleoprotein. Initially, four controls were tested. In control 1, SA-PE solution, prepared in SA-PE diluent, was flowed over the coupled particles at a concentration of 3 µg mL^{-1} for 5 min. For control 2,

detection antibody was flowed for 20 min at a concentration of 5 µg mL^{-1}, followed by SA-PE incubation for 5 min at 3 µg mL^{-1} to generate the signal. In control 3, secondary and detection antibody were flowed sequentially for 20 min at a concentration of 3 and 5 µg mL^{-1}, respectively, followed by SA-PE incubation for 5 min at 3 µg mL^{-1}. For control 4, nucleoprotein was diluted in LC buffer and flowed for 30 min at a concentration of 10 ng mL^{-1}, followed by incubation with detection antibody for 20 min at a concentration of 5 µg mL^{-1} and SA-PE for 5 min at 3 µg mL^{-1}. All antibody solutions were diluted in LowCross (LC) buffer. In between the different incubation steps and at the end of the assay, a short washing step of 1 min in storage buffer was introduced. In the second part of the optimization, nucleoprotein was incubated with the µPs for 30 min at four different concentrations (0, 0.1, 1 and 10 ng mL^{-1}). Next, the secondary antibody was flowed for 20 min at 3 µg mL^{-1}. Then, 1 and 5 µg mL^{-1} of detection antibody was flowed for 10 and 20 min in separate channels. Short washing steps were introduced in between all incubations and at the end of the assay. The µPs were visualized using both bright field and fluorescent imaging. The bright field images were used for decoding and the average fluorescence intensity for each population was calculated using the supplied software. All tests were performed at 300 mbar pressure difference between inlet and outlet wells, which corresponds to a flow rate of approximately 30 nL s^{-1} (i.e. 36 µL sample consumption per channel for 20 min incubation time)[20]. Visualization of the fluorophore was achieved using the green laser (excitation at 532 nm, laser power 60 mW, exposure time of 150 ms) and CMOS camera (10x objective) present on-board of the instrument.

2.6. Nucleoprotein detection in buffer

The interaction of the nucleoprotein with the antibody-functionalized µPs was analyzed using a sandwich assay. First, nucleoprotein with concentrations ranging between 0.026 ng mL^{-1} and 80 ng mL^{-1} was flowed over the functionalized µPs for different incubation times (15, 30 and 60 min). After incubation, the particles were washed with storage buffer for 1 min. Next, secondary antibody was introduced for 20 min at a concentration of 3 µg mL^{-1}, followed by a single short washing step. Subsequently, biotinylated detection antibody was flowed for 10 min at 1 µg mL^{-1}. All incubation steps

were performed in LC buffer. After a washing step, SA-PE solution, prepared in SA-PE diluent, was added at a concentration of 3 µg mL^{-1} and flowed for 5 min.

2.7. Nucleoprotein detection in nasopharyngeal swabs

The same sandwich assay protocol as described in section 2.6 was followed for the detection of nucleoprotein in nasopharyngeal swab eluates (in this paper referred to as nasopharyngeal swabs). Nasopharyngeal swabs, obtained from healthy donors, were eluted in 3 mL of universal transport medium and diluted 10 times in LC buffer. This solution was spiked with nucleoprotein at three different concentrations (80, 16 and 3.2 ng mL^{-1}) and incubated for 15 min.

3. Results and discussion

3.1. Signal-to-noise ratio optimization

Target recognition typically requires multiple steps in a bioassay (e.g. sandwich assay), unless a label-free detection method is used. With every new component that is being introduced in each step, the possibility of aspecific interaction between different components is increasing. Hence, it is of importance to optimize the combination of assay components in order to obtain a high signal-to-noise ratio and consequently, a low limit of detection (LOD). This is especially important when analyzing complex sample matrices.

A sandwich assay for the detection of influenza A nucleoprotein was used as a model system to conduct this study on the Evalution™ platform. Four different steps were required to generate the binding signal (Figure 1A): (1) nucleoprotein capture by the capture antibody, which was immobilized on the µP surface, sequential binding of (2) secondary antibody and (3) biotinylated detection antibody and (4) binding of SA-PE for signal generation. Cross-reactivity between the different assay components was studied by including four different controls. In control 1, SA-PE was incubated directly with the capture antibodies and the bare µP surface. In control 2, detection antibody was additionally introduced compared to control 1. All assay components except for the nucleoprotein were included in control 3, whereas in control 4, the secondary antibody was omitted. For all conditions, the signal

obtained for the three capture antibodies (Ab11, Ab12 and Ab66) was compared to the one of the non-functionalized µPs (COOH) in a multiplex analysis. The results are shown in Figure 1, together with a schematic of the assay setup for the detection of nucleoprotein.

Control 1 showed low signal for all three tested antibodies (1 a.u.), which was only slightly higher than the background signal from the µPs (0.5 a.u.), indicating negligible amount of aspecific interaction between the immobilized antibodies and the SA-PE. The signal obtained from control 2 clearly showed a high aspecific interaction between the detection antibody and the capture antibodies, whereas the introduction of the secondary antibody (control 3) and the nucleoprotein (control 4) had negligible contribution to the aspecific interaction. Furthermore, the signal of the non-functionalized µPs remained stable (1 a.u.) in all four controls, which proved that none of the assay components interacts with the µPs. This is an intrinsic advantage of the Evalution™ platform for the use in bioassay optimization as well as for target detection. Overall, the results indicated that the detection antibody was the major cause of the aspecific interaction. The difference in aspecific binding with the different capture antibodies was also observed previously for this assay when optimization was performed for standard ELISA. Importantly, the described analysis on the Evalution™ platform was finalized in 80 min (i.e. total assay time from target incubation until signal generation, washing time included), whereas the same experiments required a total assay time of at least 250 min (washing time not included) when using ELISA[21]. The short assay time, together with significantly reduced reagent consumption due to the multiplexing capacity and the microfluidic environment, demonstrated the huge potential of using Evalution™ for fast and straightforward bioassay optimization.

In an attempt to reduce the aspecific interaction of the detection antibody with the capture antibodies and thus increase the signal-to-noise ratio, the incubation time and concentration of the detection antibody were decreased. A concentration of 1 µg mL^{-1} and 10 min incubation were tested and compared to the signal obtained for 20 min and 5 µg mL^{-1}, which were used in the previous part. This

test was performed for four different nucleoprotein concentrations (0, 0.1, 1 and 10 ng mL^{-1}). The results are shown in Figure 2.

Using a high concentration of detection antibody (i.e. 5 µg mL^{-1}, Figure 2A and B) gave higher specific signals compared to the lower concentration (i.e. 1 µg mL^{-1}, Figure 2C and D). However, for the high concentration, also a higher aspecific signal (0 ng mL^{-1} of nucleoprotein) was observed as well as higher variation in the generated signal. The concentration had the biggest influence on the extend of aspecific interaction, while changing the incubation time was less effective in reducing the aspecific signal. Although a lower specific signal was obtained for the lower concentration, the signal-to-noise ratio was the highest for 1 µg mL^{-1} and 10 min incubation time (Figure 2D). Therefore, further experiments were performed using this condition. The flexibility of the Evalution™ platform to control the flow for each channel separately allowed this experiment to be performed in one run with a total assay time of 75 min.

3.2. Nucleoprotein detection in buffer

The developed sandwich-based bioassay was used to detect six different concentrations of the nucleoprotein in buffer (ranging from 0.026 ng mL^{-1} to 80 ng mL^{-1}) for 15, 30 and 60 min incubation time with the µPs. The obtained calibration curves are shown in Figure 3.

The results revealed a time dependent increase in the signal for all antibodies with the longest incubation time (60 min) giving the highest signal. The effect of the incubation time was more pronounced for Ab11 than for Ab12 and Ab66. This difference can be explained by the dissociation constants (K_D) of the antibodies. It was previously found that Ab11 had lower affinity towards the nucleoprotein (2.57 µM) compared to Ab12 (0.12 µM) and Ab66 (9.68 nM)[21], explaining the longer time that is needed to reach equilibrium. While for 15 min the behavior of the antibodies was very similar, for longer incubation times (30 and 60 min) there was a significant difference between the binding of Ab11 and the other two antibodies. Furthermore, Ab11 showed a better slope of the calibration curve (0.62) compared to the other two antibodies (0.26 for Ab12 and 0.40 for Ab66).

The LOD values have been calculated for each antibody, by interpolating from the linear calibration curves the concentration corresponding to a signal equal to three times the standard deviation of the mean background signal value. Table 1 summarizes the calculated LODs of all three capture antibodies for each condition.

Although Ab11 showed the highest sensitivity, the calculated LOD of Ab12 and Ab66 was lower for all tested incubation conditions. It is clear that, when using Ab11, low concentrations of nucleoprotein can be detected only by extending the incubation time (60 min, LOD = 0.48 ng mL^{-1}), whereas for Ab66 30 min incubation was enough to reach a substantially lower LOD (0.04 ng mL^{-1}). This can again be explained by the differences in K_D values of the antibodies. As previously published work showed, Ab66 has the lowest K_D (9.68 nM) of all three antibodies and therefore could more efficiently bind to nucleoprotein compared to Ab11 and Ab12[21]. When these antibodies were previously tested in a standard ELISA, a similar LOD value was reached for Ab66 with 60 min interaction time (0.18 ng mL^{-1}). However, a better LOD was obtained for both Ab11 and Ab12 (0.07 and 0.01 ng mL^{-1}, respectively) compared to the Evalution™ platform. In a standard ELISA, the influence of the diffusion of the analyte molecules on the interaction is not negligible (diffusion limited regime). Consequently, the K_D of the antibodies has only limited influence on the binding. On the other hand, on the Evalution™ platform, the target-receptor binding occurs in a reaction limited regime. This means the interaction depends purely on the affinity of the antibodies for their target and not on the supply of the analyte molecules[20]. Although the LOD for two antibodies was better in ELISA, the total assay time was at least 310 min (i.e. from target incubation until signal generation, washing steps not included), while the analysis performed on the Evalution™ platform had a total assay time of only 100 min (washing steps included) and a reduced reagent consumption. For instance, for analyzing one sample in triplicate for its binding to three different antibodies within 15 min, 100 µL of sample was required on Evalution™ compared to 900 µL on ELISA. This shows that the Evalution™ platform is a valuable tool for the analysis of antibody performance and can serve as an alternative for standard ELISA and SPR experiments. Moreover, by reaching similar sensitivity as a standard ELISA, especially for antibodies

with low K_D, the Evalution™ platform holds huge potential to be used as a diagnostic platform because of its shorter assay time, reduced reagent consumption, multiplexing capacity and automation.

3.3. Nucleoprotein detection in nasopharyngeal swabs

Because target detection in clinical samples is crucial, the detection of nucleoprotein on the Evalution™ platform was next tested in a relevant complex matrix. Several types of specimens are used in influenza diagnosis, such as swab, wash and aspirate originating from throat and nose. However, the most reliable detection is obtained using nasopharyngeal swabs[22]. In this study nasopharyngeal swabs obtained from healthy donors were diluted 10-fold to reduce aspecific binding[21]. The swabs were spiked with three different concentrations of recombinant nucleoprotein (3.2, 16 and 80 ng mL^{-1}), similar to the assay performed in buffer. The assay configuration was identical to the previous experiments with 15 min of incubation for nucleoprotein. The difference in the antibody performance between LC buffer and nasopharyngeal swab is shown in Figure 4.

While Ab12 showed a substantial decrease in binding in nasopharyngeal swabs, Ab11 and Ab66 were not influenced, suggesting they were more suited as bioreceptors for influenza A nucleoprotein detection. These results were also in accordance with previous findings on standard ELISA[21]. Ab11 proved to be capable of capturing nucleoprotein directly in lysis buffer (0.1 % NLS), contrary to Ab12. Therefore, it can be concluded that the binding performance of Ab12 is strongly influenced by the sample matrix and it may only have limited use as capture antibody in clinical samples. Moreover, the obtained results demonstrated the capacity of the Evalution™ platform to be used as a diagnostic system for target detection in a complex matrix.

4. Conclusions

In this work, the Evalution™ platform has been evaluated as a tool for fast and straightforward bioassay optimization in buffer and complex sample matrix. A sandwich assay for the detection of influenza A nucleoprotein was used as a model system. To obtain a high signal-to-noise ratio and a low LOD, the aspecific interactions between six different components of the sandwich assay were first

tested. The high multiplexing capacity and possibility of running up to 16 channels simultaneously allowed four different controls and the complete bioassay to be executed in a single run (80 min) for three different capture antibodies. Moreover, the reagent consumption was reduced compared to a standard ELISA. The detection antibody caused significant aspecific signals due to interaction with the capture antibodies, as also previously observed on standard ELISA[21]. Hence, the highest signal-to-noise ratio was obtained by lowering the concentration (from 5 to 1 µg mL^{-1}) and incubation time (from 20 to 10 min) of the detection antibody. Upon achieving the assay conditions giving the highest signal-to-noise ratio, different concentrations of nucleoprotein were spiked in buffer and nasopharyngeal swabs for target detection using a fast (55 min) and automated assay. Although the obtained detection limits were varying for different antibodies and different assay time, they were overall comparable to a standard ELISA. The clear effect of the assay time on the LOD when using different capture antibodies can be explained by the fact that target-receptor binding occurs in a reaction limited regime on the Evalution™ platform. Consequently, the interaction depends purely on the affinity of the antibodies for their target. The detection of nucleoprotein in nasopharyngeal swabs revealed the limited use of Ab12 as capture antibody for target detection directly in sample matrix. However, both Ab11 and Ab66 showed stable binding behavior independently on the matrix used, which was in accordance with the previous study[21]. In conclusion, obtained results reveal the Evalution™ platform as a valuable alternative for the well-established ELISA and SPR systems by allowing fast and multiplex analysis of antibody performance for bioassay optimization. Moreover, by reaching detection limits comparable to standard ELISA and allowing reliable detection in a complex sample matrix, this platform shows also huge potential to be further explored as a diagnostic tool for different application cases. Especially the short time-to-result and possibility of real-time analysis might be beneficial. Nevertheless, although up to 16 channels can be ran simultaneously, the throughput of the Evalution™ platform is limited compared to the current gold standard analysis platforms, such as ELISA. However, for several applications, throughput might not be of major importance whereas time-efficient and cost-effective

batch testing can be crucial. Hence, the Evalution™ platform might fulfill this need, especially with the recent trends in the diagnostic field showing a movement towards personalized diagnostics.

Acknowledgements

The authors would like to thank the European Union (IMI RAPP-ID), KU Leuven (Grants OT 13/058, C24/16/022 and C32/15/005) and Research Foundation−Flanders (Grants FWO G086114N and FWO G080016N) for financial support. We thank MyCartis (Belgium) for providing the Evalution™ platform, µPs and microfluidic chips. The nasopharyngeal swabs were obtained from the Laboratory of Medical Microbiology of the Vaccine and Infectious Disease Institute of the University of Antwerp.

References

[1] R.M. Lequin, Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA), Clin. Chem. 51 (2005) 2415–2418.
[2] P. Pattnaik, Surface plasmon resonance: applications in understanding receptor-ligand interaction, Appl. Biochem. Biotechnol. 126 (2005) 79–92.
[3] S.X. Leng, J.E. McElhaney, J.D. Walston, D. Xie, N.S. Fedarko, G.A. Kuchel, ELISA and multiplex technologies for cytokine measurement in inflammation and aging research, J. Gerontol. A. Biol. Sci. Med. Sci. 63 (2008) 879-884.
[4] L.L. Yin, S.P. Wang, X.N. Shan, S.T. Zhang, N.J. Tao, Quantification of protein interaction kinetics in a micro droplet, Rev. Sci. Instrum. 86 (2015) 114101.
[5] J. Pollet, F. Delport, K.P.F. Janssen, D.T. Tran, J. Wouters, T. Verbiest, J. Lammertyn, Fast and accurate peanut allergen detection with nanobead enhanced optical fiber SPR biosensor, Talanta. 83 (2011) 1436–1441.
[6] K. Knez, K.P.F. Janssen, J. Pollet, D. Spasic, J. Lammertyn, Fiber-optic high-resolution genetic screening using gold-labeled gene probes, Small. 8 (2012) 868–872.
[7] J. Lu, T. Van Stappen, D. Spasic, F. Delport, S. Vermeire, A. Gils, J. Lammertyn, Fiber optic-SPR platform for fast and sensitive infliximab detection in serum of inflammatory bowel disease patients, Biosens. Bioelectron. 79 (2016) 173–179.
[8] K.C. Neuman, A. Nagy, Single-molecule force spectroscopy: optical tweezers, magnetic tweezers and atomic force microscopy, Nat. Methods. 5 (2008) 491–505.
[9] M. Jerabek-Willemsen, T. André, R. Wanner, H.M. Roth, S. Duhr, P. Baaske, D. Breitsprecher, MicroScale Thermophoresis: Interaction analysis and beyond, J. Mol. Struct. 1077 (2014) 101–113.
[10] Luminex Corporation, xMAP Technology, (2016). www.luminexcorp.com (accessed August 2, 2016).
[11] RayBiotech Inc, Antibody Arrays, (2016). www.raybiotech.com (accessed August 2, 2016).
[12] Quansys Bioscience, Multiplexed ELISA, (2016). www.quansysbio.com (accessed August 2, 2016).
[13] T.G. Henares, S. ichi Funano, S. Terabe, F. Mizutani, R. Sekizawa, H. Hisamoto, Multiple enzyme linked immunosorbent assay system on a capillary-assembled microchip integrating valving and immuno-reaction functions, Anal. Chim. Acta. 589 (2007) 173–179.
[14] J. Moorthy, G. a Mensing, D. Kim, S. Mohanty, D.T. Eddington, W.H. Tepp, E. a Johnson, D.J. Beebe, Microfluidic tectonics platform: A colorimetric, disposable botulinum toxin enzyme-

linked immunosorbent assay system, Electrophoresis. 25 (2004) 1705–1713.
[15] H. Vaisocherova, V.M. Faca, A.D. Taylor, S. Hanash, S. Jiang, Comparative study of SPR and ELISA methods based on analysis of CD166/ALCAM levels in cancer and control human sera, Biosens. Bioelectron. 24 (2009) 2143–2148.
[16] S. Kumbhat, K. Sharma, R. Gehlot, A. Solanki, V. Joshi, Surface plasmon resonance based immunosensor for serological diagnosis of dengue virus infection, J. Pharm. Biomed. Anal. 52 (2010) 255–259.
[17] D. Hu, S.R. Fry, J.X. Huang, X. Ding, L. Qiu, Y. Pan, Y. Chen, J. Jin, C. McElnea, J. Buechler, X. Che, M.A. Cooper, Comparison of surface plasmon resonance, resonant waveguide grating biosensing and enzyme linked immunosorbent assay (ELISA) in the evaluation of a dengue virus immunoassay, Biosensors. 3 (2013) 297–311.
[18] Infliximab ELISA, (2014). http://www.apdiagroup.com/ (accessed August 15, 2016).
[19] S.K. Vashist, P.B. Luppa, L.Y. Yeo, A. Ozcan, J.H.T. Luong, Emerging Technologies for Next-Generation Point-of-Care Testing, Trends Biotechnol. 33 (2015) 692–705.
[20] D. Falconnet, J. She, R. Tornay, E. Leimgruber, D. Bernasconi, L. Lagopoulos, P. Renaud, N. Demierre, P. van den Bogaard, Rapid, Sensitive and Real-Time Multiplexing Platform for the Analysis of Protein and Nucleic-Acid Biomarkers, Anal. Chem. 87 (2015) 1582–1589.
[21] K. Leirs, P. Tewari Kumar, D. Decrop, E. Pérez Ruiz, P. Leblebici, B. Van Kelst, G. Compernolle, H. Meeuws, L. Van Wesenbeeck, O. Lagatie, L. Stuyver, A. Gils, J. Lammertyn, D. Spasic, Bioassay development for ultrasensitive detection of influenza A nucleoprotein using digital ELISA, Anal. Chem. 88 (2016) 8450–8458.
[22] M. Forman, A. Valsamakis, Specimen Collection, Transport, and Processing: Virology, in: J. Versalovic, K. Carroll, G. Funke, J. Jorgensen, M. Landry, D. Warnock (Eds.), Man. Clin. Microbiol., 10th ed., ASM Press, 2011.

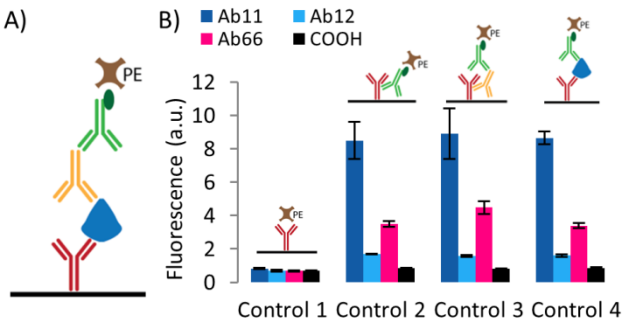

Figure 1: A) Assay layout for the detection of nucleoprotein (blue) with capture antibody (red) immobilized on the µP surface. Signal was generated by sequentially introducing a secondary antibody (yellow), biotinylated detection antibody (green) and SA-PE (brown). B) Aspecific interaction between the capture antibodies Ab11 (dark blue), Ab12 (light blue) and Ab66 (pink) and other assay components. Error bars are standard deviations of three repetitions.

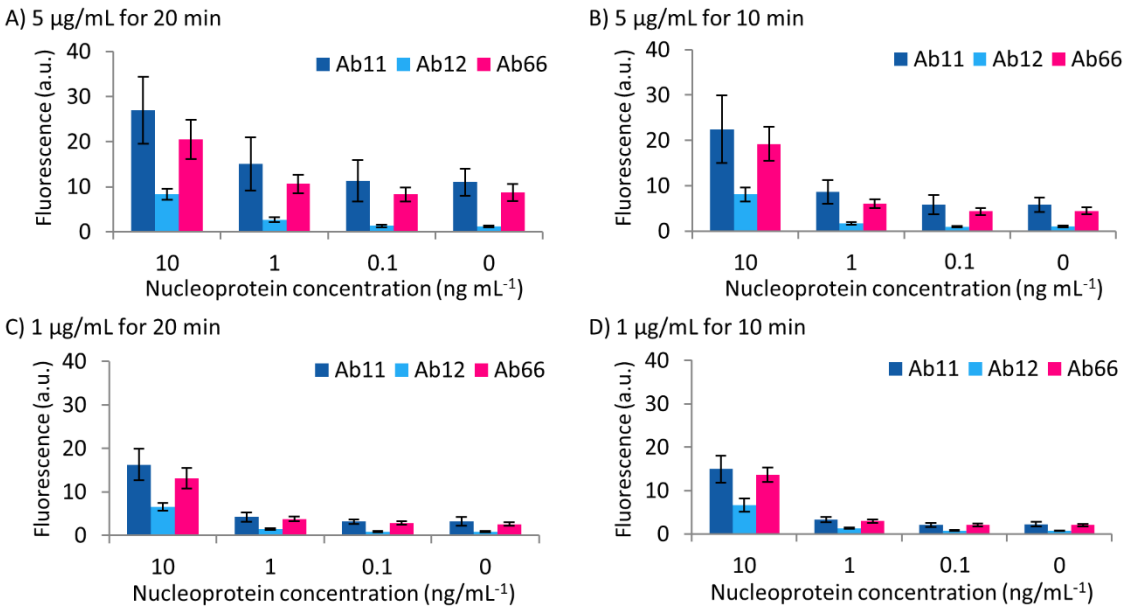

Figure 2: Comparison of the different incubation conditions for the detection antibody A) 5 µg mL^{-1} for 20 min, B) 5 µg mL^{-1} for 10 min, C) 1 µg mL^{-1} for 20 min and D) 1 µg mL^{-1} for 10 min. Nucleoprotein (0.1, 1 and 10 ng mL^{-1}) was incubated for 30 min. The results are shown for Ab11 (dark blue), Ab12 (light blue) and Ab66 (pink). Error bars are standard deviation of the average signal obtained for all µPs per antibody population.

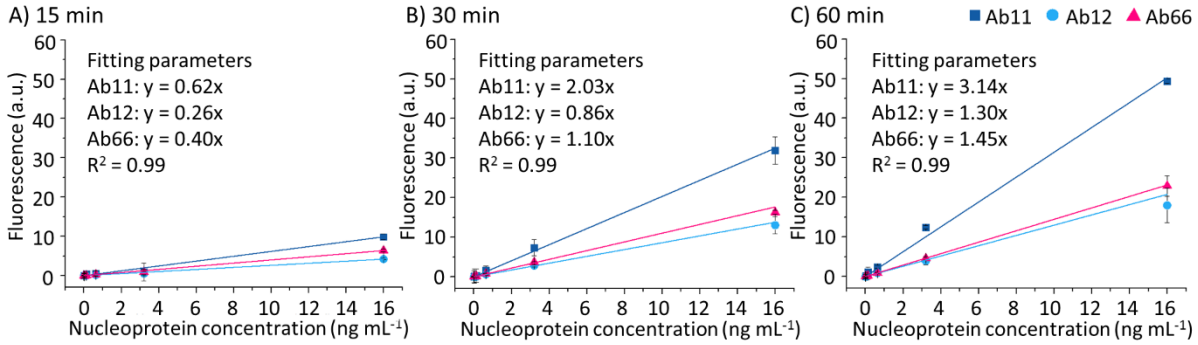

Figure 3: Calibration curves of nucleoprotein detection in LC buffer for A) 15 min, B) 30 min and C) 60 min incubation time with the capture antibodies (Ab11 (dark blue), Ab12 (light blue) and Ab66 (pink)). Five-fold dilutions of the nucleoprotein were tested, starting from 80 ng/mL, of which the linear part (0.026 ng mL^{-1} to 16 ng mL^{-1}, i.e. five different concentrations) is shown here. The fluorescent signal was corrected for the background signal of the respective antibodies (i.e. 8.54 ± 1.17 for Ab11, 1.92 ± 0.12 for Ab12 and 0.75 ± 0.95 for Ab66). Error bars are standard deviation of three repetitions.

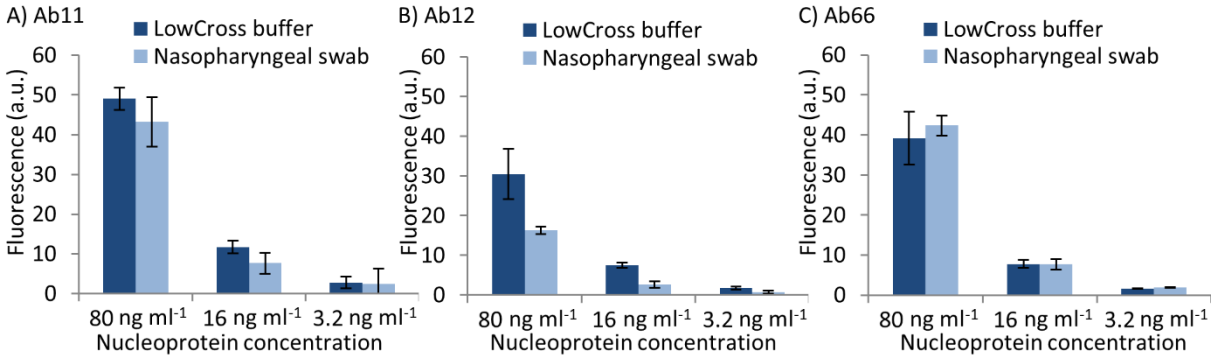

Figure 4: Nucleoprotein detection in nasopharyngeal swabs (light blue bar) and LC buffer (dark blue bar) for A) Ab11, B) Ab12 and C) Ab66. Swabs of healthy people were diluted 10 fold in LC buffer and spiked with different concentrations of recombinant nucleoprotein (80, 16 and 3.2 ng mL^{-1}). Incubation time for nucleoprotein was 15 min. Error bars are standard deviations of three repetitions.

Table 1: Limit of detection (ng mL^{-1}) for the capture of nucleoprotein with different assay conditions.

	Nucleoprotein incubation time		
Antibody	15 min	30 min	60 min
Ab11	3.04 ± 0.08	2.14 ± 0.10	0.48 ± 0.01
Ab12	0.76 ± 0.04	0.38 ± 0.09	0.26 ± 0.09
Ab66	0.60 ± 0.06	0.04 ± 0.02	0.19 ± 0.07